

Original article

Production of pure protein and antibodies and development of immunoassays to detect Ara h 3 levels in peanut varieties

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Summary Peanuts are one of the most allergenic foods and are widespread in western food products. Therefore, there has been intense research into the allergic nature of the proteins involved. Ara h 3 is one of three immunodominant allergenic proteins. It is a 60-kDa protein, which forms following cleavage of the preprotein and association of the resultant 40- and 20-kDa subunits. The large subunit has been shown to harbour most of the reactive epitopes and has the protein fold likely responsible for its trypsin inhibitor activity. In this work, we have developed a method for the high-level expression and purification of recombinant Ara h 3 40-kDa subunit. Specific antibodies have been produced and applied to the secondary and tertiary screens of hundreds of peanut cultivars. Several of these cultivars were identified that have significantly reduced accumulation of Ara h 3.

Introduction

Food allergens are a significant worldwide public health issue. Estimates for the prevalence of food allergies are around 1–2% of the total population and up to 8% of children, although the prevalence may vary between populations and age groups (Ortolani *et al.*, 2001; Woods *et al.*, 2002; Mills *et al.*, 2007). The severity of allergic reactions can vary from symptoms ranging from mild urticaria (skin reaction) to potentially lethal anaphylactic shock. Currently, the only effective treatment for food allergy is the avoidance of the allergen-containing food. Peanuts are among the most common causes of immediate hypersensitivity reactions to foods, but for peanut-allergic individuals, total avoidance is difficult because peanuts are widely used in the food industry owing to their nutritive value, their taste and low cost. Peanut-allergic patients may react to doses of peanut as low as 10 mg, and complete avoidance can be difficult owing to cross-contamination or hidden sources of peanut products in foods (Yu *et al.*, 2006). Studies suggest that up to 75% of individuals with known peanut allergy experience reactions caused by accidental exposure (Kagan *et al.*, 2003). The latter accounts for about 59% of the reported allergy-related deaths (Sicherer & Sampson, 2007). Therefore, a significant, growing

and costly portion of the FDA's (U.S. Food and Drug Administration) food recalls have been attributable to mislabelled products that contain food allergens. Over recent years, legislation has been issued aimed at a better safeguarding of the health of allergic consumers from accidental ingestion of allergenic foods (FDA, 2006). Meanwhile, no legislation or regulatory efforts have been put in place to establish standardised methods for food allergen extract preparations that are used for purposes ranging from diagnostic skin prick test and specific immunoglobulin E (IgE) ELISA measurements to standards for detection methods.

Three seed storage proteins have been identified as the major allergens in peanuts, Ara h 1, Ara h 2 and Ara h 3, as they are recognised by IgE antibodies in the sera of a majority of allergic individuals (Burks *et al.*, 1991, 1992; Rabjohn *et al.*, 1999; respectively). Other peanut allergens include Ara h 5, Ara h 6, Ara h 7, Ara h 8 and Ara h 9 (Mittag *et al.*, 2004; Lauer *et al.*, 2009). These also react with patient sera, but to a lesser extent. Hence, considerable interest has been focused on the three immunodominant proteins. Ara h 1 is a 63-kDa glycoprotein that associates into very stable trimers (Maleki *et al.*, 2000a). Ara h 2 is a much smaller protein (17–20 kDa) that has been shown to have trypsin inhibitor activity (Maleki *et al.*, 2003). Ara h 3 is translated into a 60-kDa peptide, which is proteolytically processed into 40- and 20-kDa subunits that associate into trimers and hexamers and has trypsin

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inhibitor activity (Dodo *et al.*, 2004; Jin *et al.*, 2009). Intense research for more than two decades has revealed significant information about these proteins. The linear epitopes of each of Ara h1, Ara h2 and Ara h 3 have been revealed by SPOT analysis (Burks *et al.*, 1997; Stanley *et al.*, 1997; Rabjohn *et al.*, 1999; Rougé *et al.*, 2009). Processing further increases their recognition by IgE (Maleki *et al.*, 2000b; Maleki & Hurlburt, 2004; Mondoulet *et al.*, 2005; Schmitt *et al.*, 2010; Maleki *et al.*, 2010).

Our ongoing research is focused on further characterisation of the individual allergens and their reactive epitopes, development of improved tools and methods for the detection and diagnosis of peanut allergy and the generation of hypoallergenic peanuts and products thereof. The current study addresses all of these goals. The 40-kDa subunit of Ara h 3, which houses the four dominant linear epitopes, has been optimised for the expression in *E. coli* and purified to near homogeneity. This can be applied to the development of diagnostic tools and perhaps vaccines (reviewed in Sancho *et al.*, 2010). Polyclonal antibodies to it have been generated for screening. Importantly, peanut cultivars that accumulate low levels of Ara h 3 have been identified.

Materials and methods

Cloning and optimisation of codons for Ara h 3 expression in *E. coli*

Primers for PCR were designed that incorporated Nde I and Bam HI sites for cloning into pET9a. The sequences for all the primers used in this work are shown in Table 1. The start codon is in the Nde I primer. The Bam HI primer replaces the glycine codon at position 327 with a TAA termination signal. The resultant PCR fragment was gel-purified and cloned into pTOPO

CR2.1 following the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA) yielding pHc-25. The fidelity of the PCR was confirmed by DNA sequencing. The Ara h 3 coding region was released by the digestion of pHc-25 with Nde I and Bam HI, followed by gel purification. pET9a was digested with the same enzymes and gel-purified. The two fragments were then ligated yielding pHc-26. This plasmid was transformed into BL21(DE3)pLysS (Novagen, San Diego, CA, USA), and selection was made using chloramphenicol ($30 \mu\text{g mL}^{-1}$) and kanamycin ($25 \mu\text{g mL}^{-1}$). Small cultures of several clones were tested for isopropyl β -D-thiogalactoside (IPTG)-inducible expression and analysed by SDS-PAGE. None of them showed any inducible proteins. Analysis of codon usage revealed some codons that were very rarely used in *E. coli*. Therefore, site-directed mutagenesis was used to change those codons to ones preferred by *E. coli*. Specifically, R91, R110, L112 and T183 were targeted. The Quick-Change Mutagenesis system (Stratagene, La Jolla, CA, USA) was used with the primers described in Table 1. The codons that were changed are underlined. Following mutagenesis and sequence confirmation, expression was tested again and found to be at much higher levels.

Expression and purification of recombinant Ara h 3 40-kDa subunit

Optimal expression was established by growing a 100-mL culture in 500-mL baffles flasks with LB-chloramphenicol-kanamycin at 37°C with vigorous shaking. When the culture reach an optical density of 0.6 at 600 nm, IPTG was added to a final concentration of 1 mM. One-millilitre samples were removed every hour for 5 h and again after overnight induction. Samples were resolved by SDS-PAGE and stained with GelCode Blue (Pierce, Rockford, IL, USA). Maximal expression was achieved after 4 h. One-litre cultures were then grown and induced. All of the following steps were either on ice or at 4°C . Cells were lysed in SJM buffer (Tris-Cl, pH 8.3, 10 mM DTT, 5 mM EDTA, 1 mM PMSF) plus 200 mM NaCl (SJM-200) at 5 mL g^{-1} cell paste. It was followed by sonication until all viscosity was eliminated. The lysate was cleared by centrifugation at $36,915 \text{ g}$ for 20 min. The soluble and insoluble fractions were resolved by SDS-PAGE and stained. All of the Ara h 3 was found to be insoluble (Fig. 1). In previous work, we found that growth at low temperature combined with osmotic shock resulted in more soluble protein (Koenig *et al.*, 2004). This approach did not work with Ara h 3. To determine whether the insoluble Ara h 3 was aggregated protein and not inclusion bodies, different low concentrations of chaotropic agents (urea, deoxycholate) were included in the SJM-200 buffer during lysis of the cells. In addition,

Table 1 Oligonucleotides used for mutagenesis and cloning

Site 1 mut top
cct ggt tgt cct <u>cgt</u> cac tat gaa gag cct cac aca caa ggt cgt cga
Site 1 mut bottom
tcg acg acc ttg tgt gtg agg atc ttc ata gtg <u>acg</u> agg aca acc agg
site 2-3 mut top
aa aga cca cca <u>cgt</u> <u>cgt</u> cgt cgt cca gga gaa
site 2-3 mut bottom
ttc tcc ttg <u>cag</u> <u>acg</u> <u>acg</u> tgg tgg tct tt
site 4 mut top
ttg gct ggg aac <u>acc</u> gag caa gag ttg
site 4 mut bottom
caa ctc ttg ctc <u>ggt</u> gtt ccc agc caa
nde5prime
taa gaa gga gat ata cat atg gct agc ttc cgg ca
bam3prime
ttg gct ggg aac acc gag caa gag ttg

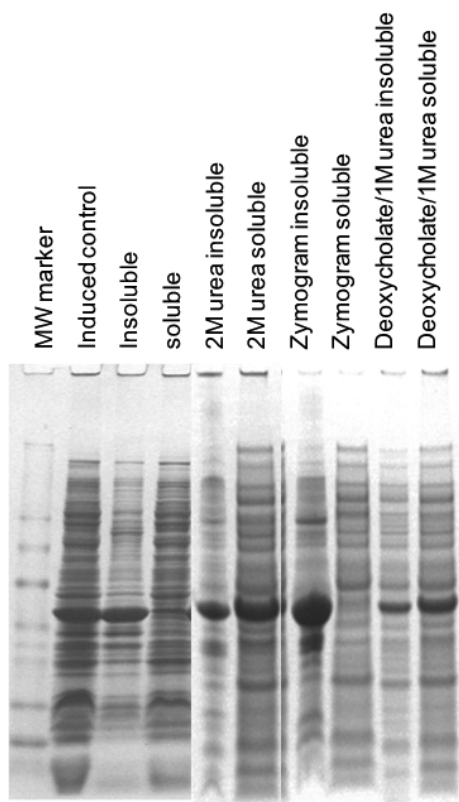


Figure 1 SDS-PAGE analysis of samples from expression and solubilisation testing. Cells were harvested by centrifugation, and the pellets were lysed in SJM-200 buffer plus the chaotropic agents indicated. Soluble and insoluble fractions were separated by centrifugation and resolved by SDS-PAGE. Proteins were revealed by staining with GelCode Blue.

substitution of SJM buffer with Zymogram renaturing buffer (Invitrogen) was tested. The solubility of Ara h 3 for each of these tests was examined by SDS-PAGE (Fig. 1). Various ion exchange columns and conditions were examined. The following yields nearly pure protein with one chromatography step. The soluble protein in SJM-200-2 M urea was diluted 1:10 with SJM-0 pH 6.5 and applied to a 50-mL MacroPrep High-S column (BioRad, Hercules, CA, USA). The column was washed with SJM-50, pH 6.5, until the absorbance at 280 nm was zero and then by a linear gradient of SJM-50 – SJM-1000. Fractions across the gradient were analysed by SDS-PAGE and stained.

Polyclonal antibody production and purification

Purified Ara h 3 was used as the antigen by Sigma Genosys (St. Louis, MO, USA) to render chicken antibodies. Antibodies were purified from the yolks by homogenisation with an equal volume of dH₂O and

freezing at -20°C for at least 12 h. The thawed mixture was centrifuged for 30 min at 29,532 g, and the aqueous fraction was collected. Ammonium sulphate was added to a final concentration of 50% and stirred for 1 h. The precipitate is collected by centrifugation and dissolved in water. Four volumes of acetone were added and chilled to -20°C for 3 h. The precipitate is collected by centrifugation, and the pellet is washed with acetone three times, followed by air drying. Pellets were stored at -80°C . Working solutions of $200\text{ }\mu\text{g mL}^{-1}$ were made when needed.

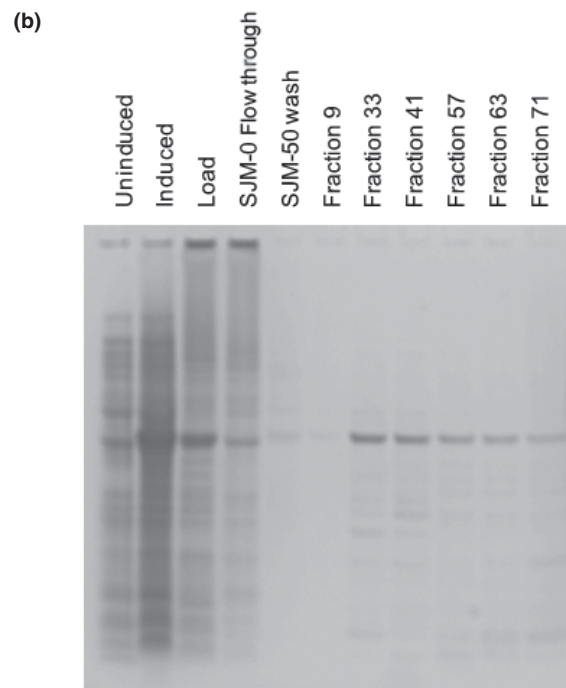
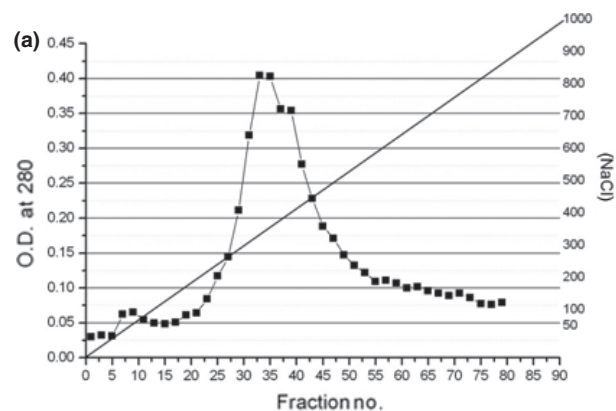


Figure 2 Ion exchange chromatography with High-S resin (BioRad, Hercules, CA, USA). (a) Fractions were assayed for protein concentration by absorbance at 280 nm (left axis). The salt gradient is shown as the diagonal line and corresponds to the concentrations (right axis). (b) SDS-PAGE of selected fractions from the chromatography.

Western blots

Western blot analysis was used to check the specificity of the antibodies, as well as confirmation of SDS-PAGE screening results of peanut cultivars. Briefly, protein samples (1 μg per lane for pure protein and 20 μg per lane for peanut extracts) were resolved on gels and electroblotted to polyvinylidene fluoride membranes. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in phosphate-buffered saline (PBST, 0.05% Tween-20) followed by chicken anti-Ara h 3 (1:5000). After washing, antibody binding was detected with horseradish peroxidase (HRP)-conjugated anti-chicken IgG (1:100,000) in 2% non-fat dry milk in PBST and ECL chemiluminescence following the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA). A FujiFilm LAS-1000 recorded the signal.

Screening peanut cultivars for reduced Ara h 3 accumulation

The peanuts that were screened were obtained from the North Carolina State University (NCSU) germplasm collection. They were derived from the irradiation of a single pure-line cultivar NC-4. Whole peanut extract (WPE) was prepared as described in Schmitt *et al.* (2010). Total protein concentration was determined by BCATM protein assay (Pierce). Equal amounts of total protein (20 μg per lane) were analysed. NC-4 was used

as the control. Proteins were resolved by SDS-PAGE and stained with GelCode Blue (Pierce). Samples that demonstrated reduced abundance were further examined by western blot and competitive ELISA. For this last technique, the assay was developed as described by Schmitt *et al.* (2004). Pure Ara h 3 was diluted to 0.5 or 1 $\text{ng } \mu\text{L}^{-1}$ in 0.1 M NaHCO_2 (pH 9.6), respectively. To every well of the 96-well ELISA plates (Immulon 4 HBX; VWR Scientific, Chester, PA, USA) except the three used for non-coated control, 50 μL of the Ara h 3 dilutions were added. The plates were then incubated for 1 h at 37 °C, followed by three washes using PBST on an automated plate washer. In order to block non-specific binding sites, 200 μL of 3% BSA were added to every well on the ELISA plates and incubated for 1 h at 37 °C. Peanut samples with unknown levels of Ara h 3 were diluted to a total protein concentration of 400 ng mL^{-1} . Equal volumes of anti-Ara h 3 antibody (1:5000 dilution) were combined with either the WPE standards or the test samples and incubated for 30 min at 37 °C to allow any Ara h 3 present in the sample to bind to the antibody. After this incubation, 50 μL of each mixture was added in triplicate wells to the ELISA plate 37 °C for 1 h followed by another wash step. Fifty microlitres of a 1:10,000 dilution of the HRP-conjugated goat anti-chicken IgG (Sigma Chemical Company, St. Louis, MO) was incubated at 37 °C for 1 h followed by more washes and development with HRP substrate TMB. Absorbance at 450 nm was determined with a Tecan Sunrise reader. (Tecan, Mannedorf, Switzerland).

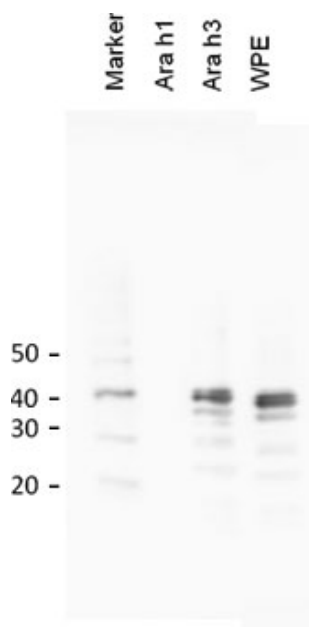


Figure 3 Western blot analysis with polyclonal antibodies against Ara h 3 40-kDa subunit. Marker = MagicMark XP (Invitrogen), Ara h 1 and Ara h 3 were purified from whole peanut, WPE = whole peanut extract.

Results

Expression and purification of Ara h 3 40-kDa subunit

The large subunit of Ara h 3 houses all of the immunodominant linear epitopes identified with SPOT analysis (Rabjohn *et al.*, 1999; Rougé *et al.*, 2009) and most of those described by Shreffler *et al.* (2004) using microarrays. Initially, an *E. coli* expression construct was generated using the original cDNA clone as a template (Rabjohn *et al.*, 1999) and pET9a as the vector. The primers used for PCR added the three N-terminal amino acid codons missing from the clone and terminated translation at codon 327. Expression from this construct was undetectable. Double-stranded mutagenesis was subsequently applied to change four codons from those rarely used in *E. coli* to those that are highly used. The resultant plasmid expressed Ara h 3 at high levels when induced with IPTG (Fig. 1). Maximal expression was found to occur within four hours. However, following lysis, the majority – if not all – of the Ara h 3 was found in the insoluble pellet. Several chaotropic agents were added to the lysis buffer and tested for the ability to release soluble Ara h 3 from the pellets. Urea and deoxycholate were

successful, and Zymogram buffer was not. Figure 1 shows an SDS-PAGE analysis of soluble and insoluble fractions of lysis of *E. coli* overexpressing Ara h 3. The 2 M urea was found to release the highest amount, and subsequent removal of the urea did not result in the precipitation of the protein. Because such a low concentration of urea worked, it is most likely that the insoluble protein was correctly folded aggregates, rather than improperly folded inclusion bodies. Among various ion exchange resins that were tested with different buffers and pHs, the best one for Ara h 3 purification was High-S (Bio-Rad, Hercules, CA, USA) for chromatography at low pH (Fig. 2). Most of the complex mixture of proteins do not bind the resin, and the Ara h 3 elutes as a nearly pure protein at approximately 400 mM NaCl.

Antibody to Ara h 3 40-kDa subunit

Polyclonal antibodies to the purified protein were raised in chickens. Figure 3 demonstrates the specificity of these antibodies. Western blot analysis was used with Ara h 1 (as negative control) and Ara h 3, which were purified from whole peanut, as well as WPE. Specific binding was observed to only the 40-kDa subunit of Ara h 3 both as purified protein and in the context of the mixture of proteins from whole peanuts.

Screening variant peanut cultivars for the reduced expression of Ara h 3

In our efforts to generate hypoallergenic peanut cultivars, a collection of mutagenised strains derived from

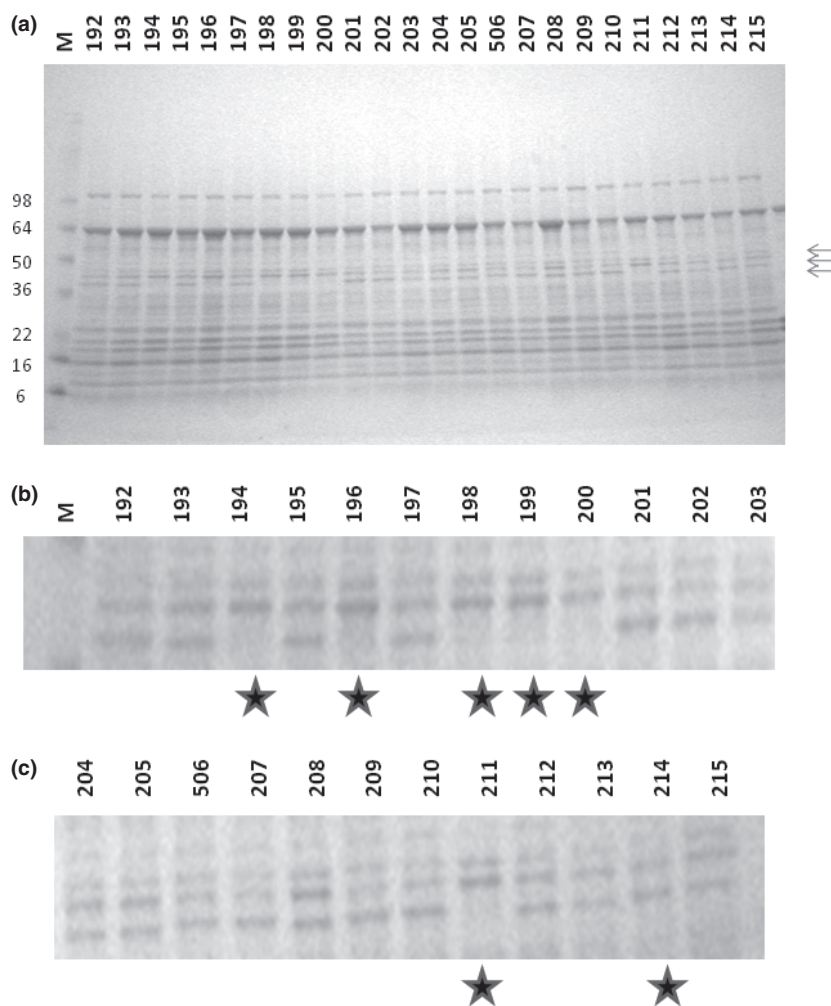


Figure 4 SDS-PAGE of whole peanut extracts of representative peanut cultivars. The cultivars are indicated by numbers. (a) Full-size gel. The arrows indicate the three isoforms of Ara h 3 40-kDa subunit. (b, c). Enlarged region of full-size gel showing only the Ara h 3 bands. Stars indicate cultivars with reduced Ara h 3.

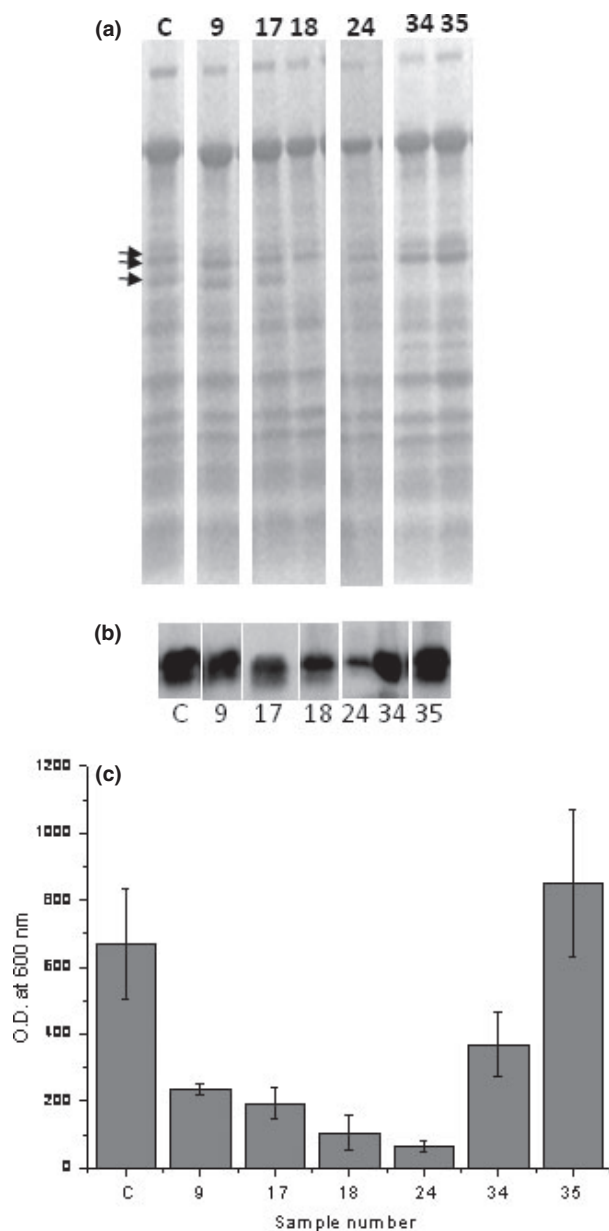


Figure 5 A. SDS-PAGE of whole peanut extracts (WPE) from cultivars with reduced accumulation of Ara h 3. Ara h 3 isoforms are indicated by arrows. The cultivars are indicated by numbers. 'C' indicates the control NC-4 variety. B. Western blot analysis of WPEs of peanut cultivars with reduced accumulation of Ara h 3. The cultivars are indicated by numbers. 'C' indicates the control NC-4. C. ELISA analysis of WPEs. The cultivars are indicated by numbers. 'C' indicates the control NC-4. Standard deviation is shown.

NC-4, a Virginia bunch type cultivar, is being screened for the presence of the three major allergens. Here, we describe our results for Ara h 3. Over 300 variants have been screened to date. Whole peanut extracts were resolved by SDS-PAGE and stained. In Fig. 4 is shown

a representative gel. The three isoforms of Ara h 3 are indicated with arrows. In this gel, the fastest migrating isoform appears to be missing or reduced in samples 194, 196, 199, 200 and 212. The slowest migrating isoform appears to be missing or reduced in samples 209, 210 and 213. The second level of screening, western blot, was only used on varieties that looked promising on stained SDS gels. In Fig. 5a is shown an SDS-PAGE analysis of cultivars with a range of Ara h 3 levels, some of which looked promising in the primary screen. Ara h 3 is indicated with the arrows. In Fig. 5b is shown western blot analysis of the same cultivars. The two slowest migrating isoforms appear as the upper band. Competitive ELISA was employed as a tertiary screen. Representative results of moderate to low accumulating stains are shown in Fig. 5c. At only 6% of the control, strain 24 has the lowest level of Ara h 3 and the other stains with the exception of 35 accumulate between 20 and 60% of the control amount.

Discussion

Food allergy is a major health problem worldwide. In particular, allergy to peanuts is problematic because of its prevalence, severity of reaction and abundance of peanut products in food. Three major allergens have been identified (Ara h 1, Ara h 2, Ara h 3) and studied intensely. Nevertheless, there is still critical information about the allergenic reaction to these proteins that remains unknown. In the work described here, we have developed a method for high-level expression and purification of the 40-kDa subunit of recombinant Ara h 3, in part, to produce highly specific antibodies and immunoassays. Specific immunoassays such as western blot and ELISA were developed and assessed in a large-scale screening project to find peanut varieties with reduced allergenic potential. The anti-Ara h 3 antibody was utilised to screen over 300 peanuts varieties, and several cultivars were identified with reduced levels of Ara h 3. These plants with reduced levels of Ara h 3 were not genetically manipulated, and the seeds are able to grow and produce more seed towards maintenance of the germplasm collection.

Ara h 3 is initially expressed as a 60-kDa polypeptide. Post-translational cleavage yields 40- and 20-kDa proteins, which associate to form a complex. These complexes further associate to form trimers and then hexamers (Jin *et al.*, 2009). Separation of the two subunits appears to be a requirement for the association of hexamers (Jin *et al.*, 2009). Other proteolytic truncations ranging from 13 to 45 kDa have been reported for the acidic subunit of Ara h 3 (Piersma *et al.*, 2005). As a result of sequence heterogeneity at two positions, it was concluded that multiple genes encode for variants of Ara h 3 (Piersma *et al.*, 2005). In the initial characterisation of Ara h 3, Rabjohn *et al.* (1999) found that there

were four dominant IgE binding sites and all were found in the amino terminal large peptide. In addition, the trypsin inhibitor region is also in that subunit (Jin *et al.*, 2009). Having pure protein will allow further detailed analysis of the allergenic and inhibitory activities of this subunit. However, more recent studies report a dominant epitope in the smaller subunit (Restani *et al.*, 2005; Rougé *et al.*, 2009). This discrepancy probably is the result of the geographic location difference where the studies were performed. The Rabjohn study used sera collected from patients in the central USA, whereas the Restani and Rougé work utilised sera from Europe.

Recombinant allergens, their subunits and antibodies will be utilised towards structural, biochemical and immunological characterisations to develop better detection, diagnosis and therapeutic tools. It has been shown that recombinant allergens that are mutated to reduce IgE binding can be used for therapeutic desensitisation of allergic individuals (reviewed in Valenta & Niederberger, 2007; Nowak-Węgrzyn, 2007). One study reported that the recombinant peanut allergens, Ara h 1, Ara h 2 and full-length Ara h 3 can be used diagnostically to monitor reactivity to peanuts before and after desensitisation (Moneret-Vautrin *et al.*, 2008). We suggest that the use of the most relevant allergen isoforms or subunits of allergens is important for improved accuracy of diagnostic tests by avoiding non-specific antibody binding.

The purified recombinant Ara h 3 protein was used to produce highly specific anti-Ara h 3 antibody, which was shown to recognise Ara h 3 in peanut. No other proteins in the peanut extracts, including purified Ara h 1 (used as negative control), were recognised by this antibody. SDS-PAGE was used to examine approximately 300 peanut varieties from the NCSU germplasm collection. Promising varieties were examined by western blot, and the amount of Ara h 3 was quantified by competitive ELISA. Importantly, we identified several peanuts that had reduced levels of the 40-KDa Ara h 3 isoforms. These results and methods of screening can have promising uses in the future (Riascos *et al.*, 2010). We have previously developed antibodies and immunoassays against the major peanut allergens Ara h 1 and 2 (Schmitt *et al.*, 2004). Along with this new antibody, we plan to screen a large number of peanut cultivars from the US germplasm collection to find peanuts with reduced levels of different allergens. Peanut varieties missing one allergen will then be bred with a peanut missing a different allergen to develop peanuts with reduced allergenic potential. Although protease inhibitors have been thought to provide disease resistance to plants (Ryan, 1990), inhibiting accumulation of Ara h 2 and Ara h 6 had no detrimental effects with respect to the growth of the pathogen *Aspergillus flavus* (Chu *et al.*, 2008). It is important to note that these experiments are at the preliminary stages and breeding a

hypoallergenic plant can take up to 10 years or longer to accomplish, so future marketability of the reduced allergenicity peanuts is not clear at this point.

Acknowledgments

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